Inhibition of inositol trisphosphate-induced calcium release by cyclic ADP-ribose in A7r5 smooth-muscle cells and in 16HBE14o- bronchial mucosal cells

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Ca²⁺ release from intracellular stores occurs via two families of intracellular channels, each with their own specific agonist: Ins(1,4,5)P₃ for the Ins(1,4,5)P₃ receptor and cyclic ADP-ribose (cADPR) for the ryanodine receptor. We now report that cADPR inhibited Ins(1,4,5)P₃-induced Ca²⁺ release in permeabilized A7r5 cells with an IC₅₀ of 20 μM, and in permeabilized 16HBE14o-bronchial mucosal cells with an IC₅₀ of 35 μM. This inhibition was accompanied by an increase in specific [³H]Ins(1,4,5)P₃ binding. 8-Amino-cADPR, but not 8-bromo-cADPR, antagon-

ized this effect of cADPR. The inhibition was prevented by a whole series of inositol phosphates (10 μM) that did not affect Ins(1,4,5)P₃-induced Ca²⁺ release, and by micromolar concentrations of PP_i and various nucleotide di- or triphosphates. We propose that cADPR must interact with a novel regulatory site on the Ins(1,4,5)P₃ receptor or on an associated protein. This site is neither the Ins(1,4,5)P₃-binding domain, which prefers Ins(1,4,5)P₃ and only binds nucleotides and PP_i in the millimolar range, nor the stimulatory adenine nucleotide binding site.

INTRODUCTION

Many extracellular signals control intracellular processes via changes in intracellular Ca²⁺ concentration. Both the entry of extracellular Ca²⁺ and the release of Ca²⁺ from the intracellular stores contribute to the rise in intracellular [Ca²⁺]. In most cell types, Ca²⁺ release from the stores occurs through one or both of two closely related Ca²⁺ channels: the Ins(1,4,5)P₃ receptor and the ryanodine receptor (RyR) [1–8]. Ins(1,4,5)P₃ [2] and cyclic ADP-ribose (cADPR) [9,10] have so far been considered as being specific agonists for the Ins(1,4,5)P₃ receptor and the RyR respectively. In order to investigate a possible cross-talk between these two Ca²⁺-release mechanisms, we have studied the effect of cADPR on Ins(1,4,5)P₃-induced Ca²⁺ release in permeabilized A7r5 smooth-muscle cells, and in 16HBE14o-bronchial mucosal cells. We now report that cADPR also interacts with the Ins(1,4,5)P₃ receptor and inhibits Ins(1,4,5)P₃-induced Ca²⁺ release. This effect involves a novel regulatory site that can also bind PP_i-containing nucleotides and various inositol phosphate analogues that by themselves do not mobilize Ca²⁺. cADPR may therefore function as a unique messenger for fine tuning of the balance between Ins(1,4,5)P₃- and ryanodine-sensitive Ca²⁺ release.

MATERIALS AND METHODS

A7r5 cells, an established cell line derived from embryonic rat aorta, were used between the 7th and the 18th passage after receipt from the American Type Culture Collection (Bethesda, MD, U.S.A.) and subcultured weekly by trypsinization. The cells were cultured at 37 °C in a 9% CO₂ incubator in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal-calf serum, 3.8 mM L-glutamine, 0.9% (v/v) non-essential amino

acids, 85 IU·ml⁻¹ penicillin and 85 μg·ml⁻¹ streptomycin. 16HBE14o- cells, which were derived from a bronchial surface epithelium, were cultured in a 50% (v/v)/50% (v/v) mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium, supplemented with 10% fetal-calf serum, 3.8 mM L-glutamine, 85 IU·ml⁻¹ penicillin and 85 μg·ml⁻¹ streptomycin. A7r5 cells were seeded in 12-well dishes (Costar, MA, U.S.A.; 4 cm²) at a density of approx. 10⁴ cells·cm⁻² and investigated 5–6 days later. 16HBE14o- cells were seeded at a density of approx. 2 × 10⁵ cells·cm⁻² and investigated 8 days later.

⁴⁵Ca²⁺ fluxes on monolayers of saponin-permeabilized cells at 25 °C were done as described [11]. The stores were loaded for 1 h in 120 mM KCl, 30 mM imidazole (pH 6.8), 5 mM MgCl₂, 5 mM ATP, 0.44 mM EGTA, 10 mM Na₂N₃ and 150 nM free Ca²⁺ (23 μCi·ml⁻¹) and then washed twice in efflux medium, containing 120 mM KCl, 30 mM imidazole (pH 6.8) and 1 mM EGTA. This medium (1 ml) was then added to the cells and replaced every 2 min for 20 min. Additions were as indicated in the Figures. cADPR was, unless otherwise indicated, from Amersham International (Amersham, Bucks., U.K.).

Preparation of microsomes from A7r5 cells and measurement of [³H]Ins(1,4,5)P₃ binding to those microsomes were performed essentially as described previously [12]. The standard binding buffer contained 50 mM Tris/HCl, 10 mM β-mercaptoethanol and 1 mM EDTA. Measurements were done at both pH 8.3 and pH 7.4, whereby the [³H]Ins(1,4,5)P₃ concentrations used were 3.8 nM and 5.3 nM respectively. Changes in the pH or in the composition of the binding buffer are indicated in Table 4.

RESULTS

Inhibition of the Ins(1,4,5)P₃-induced Ca²⁺ release by cADPR

Permeabilized A7r5 cells loaded to equilibrium with ⁴⁵Ca²⁺, slowly lost their accumulated ⁴⁵Ca²⁺ during incubation in efflux

Figure 1 cADPR inhibits Ins(1,4,5) P_3 -induced Ca^{2+} release in permeabilized A7r5 cells

(a) Ca^{2+} release induced by a 2 min stimulation with 1 μM Ins(1,4,5) P_3 in the absence (●) and presence (○) of 40 μM cADPR added at the time of Ins(1,4,5) P_3 addition. The application of Ins(1,4,5) P_3 is indicated by the bar above the trace. The results are expressed as fractional loss, i.e. the amount of Ca^{2+} leaving the stores in 2 min, divided by the total store Ca^{2+} content at that time. (b) Ca^{2+} release induced by 1 μM Ins(1,4,5) P_3 in the presence of the indicated [cADPR]. The Ca^{2+} release induced by 1 μM Ins(1,4,5) P_3 in the absence of cADPR was taken as 100%. Typical for five experiments.

medium. A short exposure to 1 μM Ins(1,4,5) P_3 accelerated this Ca^{2+} loss (Figure 1a, solid circles). The release did not occur if 40 μM cADPR was added at the time of Ins(1,4,5) P_3 addition (Figure 1a, open circles). The IC_{50} for inhibiting Ins(1,4,5) P_3 -induced Ca^{2+} release under these conditions was 20 μM (Figure 1b).

The precursor of cADPR (NAD) and its metabolite (ADP-ribose) did not inhibit Ins(1,4,5) P_3 -induced Ca^{2+} release at a concentration of 40 μM (Table 1). We also increased their concentration up to 1 mM, but this resulted in a stimulation of the Ins(1,4,5) P_3 -induced Ca^{2+} release and not an inhibition [13]. We also tested some cADPR analogues. cGDP-ribose, at a concentration of 40 μM (Table 1) and 100 μM (results not shown), was unable to mimic the inhibitory effect of cADPR. 8-Amino-cADPR (40 μM) did not inhibit the Ins(1,4,5) P_3 -induced Ca^{2+} release, but slightly stimulated it (Table 1). 8-Bromo-cADPR (40 μM) did inhibit the release, but this effect was much smaller than that of cADPR (Table 1). 8-Amino- and 8-bromo-cADPR both antagonize the stimulation of the RYR by cADPR [14], but only 8-amino-cADPR antagonized the inhibition of the Ins(1,4,5) P_3 -induced Ca^{2+} release by cADPR (Table 1). The EC_{50} for antagonizing the inhibition of Ins(1,4,5) P_3 -induced Ca^{2+} release by 40 μM cADPR was 32 μM 8-amino-cADPR (results not shown).

Table 1 Pharmacology of the inhibition of Ins(1,4,5) P_3 -induced Ca^{2+} release by cADPR in permeabilized A7r5 cells

The effect of 40 μM of the indicated compounds on the Ca^{2+} release induced by 1 μM Ins(1,4,5) P_3 was tested by the same protocol as that in Figure 1(a). The 100% value was the Ca^{2+} release induced by 1 μM Ins(1,4,5) P_3 in the absence of any added compound. Means $\pm$ S.E.M. are shown for the indicated number of independent experiments, each performed in duplicate.

Compound	Ca^{2+} release (%)	
cADPR	4.8 $\pm$ 0.6	(n = 5)
NAD	104.2 $\pm$ 5.1	(n = 5)
ADP-ribose	120.7 $\pm$ 5.6	(n = 3)
cGDP-ribose	96.1 $\pm$ 1.4	(n = 5)
8-Amino-cADPR	128.8 $\pm$ 3.0	(n = 4)
8-Bromo-cADPR	76.3 $\pm$ 5.4	(n = 4)
cADPR + 8-amino-cADPR	114.4 $\pm$ 9.2	(n = 4)
cADPR + 8-bromo-cADPR	3.6 $\pm$ 1.5	(n = 4)

We were concerned that the observed effect of cADPR was due to the presence of an impurity in the cADPR preparation. We therefore tested the effect of cADPR from various suppliers on the Ca^{2+} release induced by 1 μM Ins(1,4,5) P_3 (Table 2). Amersham International, Molecular Probes and Research Biochemicals International all supplied cADPR as free acid. These three compounds were all effective inhibitors of Ins(1,4,5) P_3 -induced Ca^{2+} release. This finding makes it unlikely that a contaminant caused the inhibition. Calbiochem and Sigma supplied cADPR as sodium phosphate buffer salt (approx. 70%, w/w) and not as free acid. Both batches did not inhibit Ins(1,4,5) P_3 -induced Ca^{2+} release and even prevented the inhibition by cADPR from Amersham International (Table 2). The 10 mol P_i per mol of cADPR present in the latter two batches was probably responsible for this lack of effect, since also cADPR from Amersham International became ineffective in the presence of a 10-fold excess of P_i . For this reason, all subsequent experiments were performed using cADPR from Amersham International.

Table 2 Effect of cADPR from various sources on the Ins(1,4,5) P_3 -induced Ca^{2+} release in permeabilized A7r5 cells

The effect of 40 μM cADPR from the indicated source and of 400 μM P_i on the Ca^{2+} release induced by 1 μM Ins(1,4,5) P_3 was tested by the same protocol as that in Figure 1(a). Ca^{2+} release in the absence of cADPR was set at 100%. Means $\pm$ S.E.M. are shown for the indicated number of independent experiments, each performed in duplicate.

Compound	Ca^{2+} release (%)	
cADPR (Amersham)	4.8 $\pm$ 0.6	(n = 5)
cADPR (Molecular Probes)	3.0 $\pm$ 2.0	(n = 3)
cADPR (Research Biochemicals International)	7.0 $\pm$ 4.0	(n = 3)
cADPR (Calbiochem)	113.8 $\pm$ 4.7	(n = 3)
cADPR (Sigma)	110.8 $\pm$ 2.3	(n = 7)
cADPR (Amersham) + cADPR (Calbiochem)	87.6 $\pm$ 3.0	(n = 3)
cADPR (Amersham) + cADPR (Sigma)	84.1 $\pm$ 4.0	(n = 3)
cADPR (Amersham) + P_i	88.7 $\pm$ 5.8	(n = 4)

Figure 2 cADPR inhibits $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release in permeabilized 16HBE140 cells

(a) Ca^{2+} release induced by a 2 min stimulation with 3 μM $\text{Ins}(1,4,5)\text{P}_3$ in the absence (●) and presence (○) of 60 μM cADPR added at the time of $\text{Ins}(1,4,5)\text{P}_3$ addition. The application of $\text{Ins}(1,4,5)\text{P}_3$ is indicated by the bar above the trace. The results are expressed as fractional loss, i.e. the amount of Ca^{2+} leaving the stores in 2 min, divided by the total store Ca^{2+} content at that time. (b) Ca^{2+} release induced by 3 μM $\text{Ins}(1,4,5)\text{P}_3$ in the presence of the indicated [cADPR]. The Ca^{2+} release induced by 3 μM $\text{Ins}(1,4,5)\text{P}_3$ in the absence of cADPR was taken as 100%. Typical for three experiments.

Inhibition by cADPR was independent of modulation of RYR activity

We [15,16] and others [17] previously presented evidence that A7r5 cells do not have functional RYRs, since they do not respond to 25 mM caffeine and ryanodine is unable to deplete their Ca^{2+} stores. However, we did some additional experiments to exclude the involvement of RYRs in the cADPR-induced inhibition of the $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release. First, addition of 40 μM cADPR in the absence of $\text{Ins}(1,4,5)\text{P}_3$ did not release Ca^{2+} in permeabilized A7r5 cells (results not shown). This finding excludes that the inhibitory effect of cADPR on $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release was due to its ability to deplete Ca^{2+} stores via RYR modulation. Secondly, the inhibitory effect of cADPR on $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release was not affected by ryanodine (0.1–100 μM) (results not shown). Thirdly, the effect of cADPR on $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release was not mimicked by ryanodine (0.1–100 μM) (results not shown). A fourth argument against the possible involvement of a RYR in the inhibition of the $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release is that cADPR also exhibited its inhibitory effect in 16HBE140- cells from bronchial mucosa (Figure 2a), which do not express RYRs (results not shown). The IC_{50} for inhibiting the Ca^{2+} release

Figure 3 Effect of cADPR on $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release in permeabilized A7r5 cells

The stores were challenged with the indicated [$\text{Ins}(1,4,5)\text{P}_3$] in the absence (●) or in the presence (○) of 40 μM cADPR. Ca^{2+} release induced by 100 μM $\text{Ins}(1,4,5)\text{P}_3$ in the absence of cADPR was taken as 100%. The results shown are typical for three independent experiments.

induced by 3 μM $\text{Ins}(1,4,5)\text{P}_3$ under these conditions was 35 μM (Figure 2b). We therefore propose that cADPR can directly inhibit $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release independently of its effect on RYRs.

cADPR does not interact with the $\text{Ins}(1,4,5)\text{P}_3$ -binding site of the $\text{Ins}(1,4,5)\text{P}_3$ receptor

Figure 3 shows the Ca^{2+} release as a function of the [$\text{Ins}(1,4,5)\text{P}_3$] in the absence (solid circles) and presence (open circles) of 40 μM cADPR in permeabilized A7r5 cells. The inhibition by cADPR only occurred at the lower $\text{Ins}(1,4,5)\text{P}_3$ concentrations, and disappeared at the higher $\text{Ins}(1,4,5)\text{P}_3$ concentrations. Although the inhibitory effect of cADPR was antagonized by $\text{Ins}(1,4,5)\text{P}_3$, it is unlikely that cADPR bound to the $\text{Ins}(1,4,5)\text{P}_3$ -binding domain of the $\text{Ins}(1,4,5)\text{P}_3$ receptor, because various inositol tris-, tetrakis-, pentakis- and hexakisphosphates (10 μM), which did not affect $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release in the absence of cADPR, also prevented the inhibition by cADPR (Table 3).

In order to more clearly establish whether or not cADPR would act as a competitive inhibitor at the $\text{Ins}(1,4,5)\text{P}_3$ -binding domain of the $\text{Ins}(1,4,5)\text{P}_3$ receptor, we have directly measured its effect on [^3H] $\text{Ins}(1,4,5)\text{P}_3$ binding to A7r5 microsomes. As expected, ATP (100 μM) potently displaced [^3H] $\text{Ins}(1,4,5)\text{P}_3$ at pH 8.3 (Table 4). In contrast, cADPR was completely ineffective when tested at the same concentration. In order to check if cADPR would only interact with the $\text{Ins}(1,4,5)\text{P}_3$ -binding site under more physiological conditions, we repeated the experiments at pH 7.4. Surprisingly, under the latter condition, cADPR (50 or 100 μM) stimulated the binding activity up to 11-fold (Table 4). P_i and L- $\text{Ins}(1,4,5)\text{P}_3$, which antagonized the effect of cADPR on Ca^{2+} release (Tables 2 and 3), also prevented the increased [^3H] $\text{Ins}(1,4,5)\text{P}_3$ binding in the presence of cADPR, without affecting [^3H] $\text{Ins}(1,4,5)\text{P}_3$ binding under control conditions in the absence of cADPR. The competitive action of ATP on [^3H] $\text{Ins}(1,4,5)\text{P}_3$ binding was not, however, affected by the changed pH conditions. These findings indicate that the observed functional effects on Ca^{2+} fluxes and the effects on [^3H]- $\text{Ins}(1,4,5)\text{P}_3$ binding were related, and exclude the possibility that cADPR acted as a competitive inhibitor for the $\text{Ins}(1,4,5)\text{P}_3$ -binding site of the $\text{Ins}(1,4,5)\text{P}_3$ receptor. [^3H] InsP_3 -binding activity was much lower at pH 7.4 compared with pH 8.3 [18],

Table 3 Effect of inositol polyphosphates on the cADPR-induced inhibition of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release in permeabilized A7r5 cells

Ca^{2+} release was induced by $1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ plus $10\ \mu\text{M}$ of the indicated inositol polyphosphate in the absence and in the presence of $40\ \mu\text{M}$ cADPR. Ca^{2+} release in the absence of added compounds was set at 100%. Means $\pm$ S.E.M. are shown for the indicated number of independent experiments, each performed in duplicate.

Compound	Ca^{2+} release (%)	
	— cADPR	+ cADPR
$1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$	100.0	4.8 ± 0.6 ($n = 5$)
$1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ + $10\ \mu\text{M}$ $\text{Ins}(2,4,5)\text{P}_3$	109.9 ± 3.2 ($n = 3$)	100.1 ± 3.8 ($n = 3$)
$1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ + $10\ \mu\text{M}$ $\text{L-Ins}(1,4,5)\text{P}_3$	97.9 ± 5.4 ($n = 3$)	74.1 ± 4.0 ($n = 3$)
$1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ + $10\ \mu\text{M}$ $\text{Ins}(1,5,6)\text{P}_3$	105.8 ± 2.5 ($n = 3$)	76.7 ± 1.8 ($n = 3$)
$1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ + $10\ \mu\text{M}$ $\text{Ins}(1,3,4)\text{P}_3$	103.4 ± 3.5 ($n = 3$)	65.4 ± 3.8 ($n = 3$)
$1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ + $10\ \mu\text{M}$ $\text{Ins}(1,3,4,5)\text{P}_4$	108.7 ± 2.5 ($n = 3$)	100.0 ± 1.6 ($n = 3$)
$1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ + $10\ \mu\text{M}$ $\text{Ins}(1,3,4,6)\text{P}_4$	102.2 ± 2.9 ($n = 3$)	96.0 ± 3.9 ($n = 3$)
$1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ + $10\ \mu\text{M}$ $\text{Ins}(1,2,5,6)\text{P}_4$	98.7 ± 5.8 ($n = 3$)	63.7 ± 3.2 ($n = 3$)
$1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ + $10\ \mu\text{M}$ $\text{Ins}(3,4,5,6)\text{P}_4$	110.7 ± 6.3 ($n = 3$)	95.3 ± 5.6 ($n = 3$)
$1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ + $10\ \mu\text{M}$ $\text{Ins}(1,3,4,5,6)\text{P}_5$	95.7 ± 5.8 ($n = 3$)	75.5 ± 2.8 ($n = 3$)
$1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ + $10\ \mu\text{M}$ $\text{Ins}(1,2,3,4,5,6)\text{P}_6$	90.6 ± 3.6 ($n = 3$)	30.8 ± 5.6 ($n = 3$)

Table 4 Effect of cADPR on $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding to A7r5 microsomes at pH 8.3 and 7.4

Effect of the indicated [cADPR] and [ATP] on the binding of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ to A7r5 microsomes in the absence and presence of $1\ \text{mM}$ P_i or $5\ \mu\text{M}$ $\text{L-Ins}(1,4,5)\text{P}_3$. The $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ concentration was $3.8\ \text{nM}$ at pH 8.3 and $5.3\ \text{nM}$ at pH 7.4. Means $\pm$ S.E.M. are shown for the indicated number of independent experiments, each performed in triplicate.

Compound	pH	$[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding (fmol/mg)	
Control	8.3	86 ± 5	($n = 4$)
cADPR ($100\ \mu\text{M}$)	8.3	89 ± 5	($n = 3$)
ATP ($100\ \mu\text{M}$)	8.3	62 ± 1	($n = 3$)
ATP ($1\ \text{mM}$)	8.3	37 ± 1	($n = 3$)
Control	7.4	17 ± 2	($n = 5$)
cADPR ($50\ \mu\text{M}$)	7.4	102 ± 6	($n = 3$)
cADPR ($100\ \mu\text{M}$)	7.4	181 ± 15	($n = 2$)
ATP ($100\ \mu\text{M}$)	7.4	9 ± 4	($n = 3$)
P_i ($1\ \text{mM}$)	7.4	17 ± 1	($n = 2$)
cADPR ($50\ \mu\text{M}$) + P_i ($1\ \text{mM}$)	7.4	18 ± 3	($n = 2$)
$\text{L-Ins}(1,4,5)\text{P}_3$ ($5\ \mu\text{M}$)	7.4	21 ± 1	($n = 2$)
cADPR ($50\ \mu\text{M}$) + $\text{L-Ins}(1,4,5)\text{P}_3$ ($5\ \mu\text{M}$)	7.4	34 ± 3	($n = 2$)

and the decrease in binding activity could only be partially compensated for by the increase in the $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ concentration present in the binding buffer. Therefore it was not possible to determine if cADPR affected the K_D and/or the B_{max} of the $\text{Ins}(1,4,5)\text{P}_3$ receptor.

cADPR does not interact with the stimulatory adenine nucleotide-binding site of the $\text{Ins}(1,4,5)\text{P}_3$ receptor

cADPR has the ability to compete with ATP for the adenine nucleotide-binding site on the cardiac RYR [19]. The $\text{Ins}(1,4,5)\text{P}_3$ receptor also contains adenine nucleotide-binding sites [20–22], which stimulate the Ca^{2+} release in the presence of $\text{Ins}(1,4,5)\text{P}_3$ [23–26]. The pharmacology of the stimulation of the $\text{Ins}(1,4,5)\text{P}_3$ receptor in A7r5 cells resembled that in other tissues, since the stimulation was specific for adenine nucleotides and since di- and triphosphates were equally effective [13]. Table 5 compares the Ca^{2+} release in response to $1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ plus $40\ \mu\text{M}$ cADPR

in a medium with $40\ \mu\text{M}$ of a nucleotide mono-, di- or triphosphate of the indicated base. The inhibition by cADPR was largely prevented by $40\ \mu\text{M}$ ATP, which is the half-maximal effective concentration for activating the stimulatory adenine nucleotide-binding site on the $\text{Ins}(1,4,5)\text{P}_3$ receptor [13]. This finding raises the possibility that cADPR may act on the stimulatory nucleotide-binding site of the $\text{Ins}(1,4,5)\text{P}_3$ receptor. cADPR did not, however, interact with this site. Firstly, this was because prevention of the inhibition by cADPR was not specific for adenine nucleotides, since ITP, GTP, CTP and UTP were as effective as ATP in counteracting the cADPR effect (Table 5). Secondly, while diphosphates were as effective as triphosphates in activating the stimulatory nucleotide-binding site [13], diphosphates were much less effective than triphosphates in counteracting the cADPR effect (Table 5). Thirdly, it was because $1\ \text{mM}$ P_i or PP_i , $40\ \mu\text{M}$ 8-amino-cADPR or $10\ \mu\text{M}$ $\text{Ins}(1,3,4,5)\text{P}_4$ all prevented the inhibition by $40\ \mu\text{M}$ cADPR, whereas they did not affect the stimulation by $40\ \mu\text{M}$ ATP (Table 6). PP_i was much more effective (EC_{50} of $2.7\ \mu\text{M}$) than P_i (EC_{50} of $210\ \mu\text{M}$) in this respect (results not shown).

cADPR and caffeine interact with different sites

Caffeine, another activator of the RYR, also inhibits the $\text{Ins}(1,4,5)\text{P}_3$ receptor [27] and this inhibition is prevented by $\text{Ins}(1,4,5)\text{P}_3$ [16,28] and ATP [16]. Caffeine did not, however, interact with the cADPR-binding site, because the inhibition by $50\ \text{mM}$ caffeine, in contrast with that by cADPR, was not affected by $1\ \text{mM}$ P_i , $1\ \text{mM}$ PP_i , $40\ \mu\text{M}$ 8-amino-cADPR or $10\ \mu\text{M}$ $\text{Ins}(1,3,4,5)\text{P}_4$ (Table 6), and was prevented only by adenine nucleotides (Table 7).

DISCUSSION

The main finding in the present work is that cADPR inhibited $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release and that the inhibition was prevented by a whole series of inositol phosphates that did not affect $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release, and by micromolar concentrations of PP_i and nucleotide di- or triphosphates. We therefore propose that cADPR must interact with a novel regulatory site on the $\text{Ins}(1,4,5)\text{P}_3$ receptor. Alternatively, cADPR may act on an associated protein, as was also proposed for the RYR [29,30]. The cADPR interaction site also binds inositol phosphates, PP_i and various nucleotide di- or tri-

Table 5 Differential effects of nucleotides on Ca^{2+} release induced by 1 μM Ins(1,4,5) P_3 in the presence of 40 μM cADPR in permeabilized A7r5 cells

Ins(1,4,5) P_3 -induced Ca^{2+} release in the presence of 40 μM cADPR and 40 μM of the indicated nucleotide triphosphate (NTP), diphosphate (NDP) or monophosphate (NMP) is expressed each time as a percentage of the Ca^{2+} release in the absence of cADPR. Means $\pm$ S.E.M. are shown for the indicated number of independent experiments, each performed in duplicate. In the absence of added nucleotides, 40 μM cADPR reduced the Ins(1,4,5) P_3 -induced Ca^{2+} release to $4.8 \pm 0.6\%$ ($n = 5$) of the control value.

Base	Ca^{2+} release (%)		
	Ins(1,4,5) P_3 + NTP	Ins(1,4,5) P_3 + NDP	Ins(1,4,5) P_3 + NMP
Adenine	88.0 ± 5.2 ($n = 3$)	54.1 ± 5.2 ($n = 3$)	15.8 ± 4.6 ($n = 3$)
Hypoxanthine	73.7 ± 2.5 ($n = 3$)	42.7 ± 1.2 ($n = 3$)	8.5 ± 2.8 ($n = 3$)
Guanine	88.7 ± 3.6 ($n = 3$)	55.7 ± 3.9 ($n = 3$)	14.9 ± 3.9 ($n = 3$)
Cytosine	79.1 ± 1.3 ($n = 3$)	34.1 ± 6.2 ($n = 3$)	9.8 ± 5.2 ($n = 3$)
Uracil	73.3 ± 3.6 ($n = 3$)	37.9 ± 5.2 ($n = 3$)	9.2 ± 1.2 ($n = 3$)

Table 6 Effect of P_i , PP_i , 8-amino-cADPR and Ins(1,3,4,5) P_4 on Ins(1,4,5) P_3 -induced Ca^{2+} release and its modulation by ATP, cADPR or caffeine in permeabilized A7r5 cells

The following concentrations were used: 1 mM P_i , 1 mM PP_i , 40 μM 8-amino-cADPR, 10 μM Ins(1,3,4,5) P_4 , 40 μM ATP, 40 μM cADPR and 50 mM caffeine. Ca^{2+} release in the presence of 1 μM Ins(1,4,5) P_3 in the absence of ATP, cADPR or caffeine, was taken as 100%. Means $\pm$ S.E.M. are shown for the indicated number of independent experiments, each performed in duplicate.

Compound	Ca^{2+} release (%)			
	Ins(1,4,5) P_3	Ins(1,4,5) P_3 + ATP	Ins(1,4,5) P_3 + cADPR	Ins(1,4,5) P_3 + caffeine
Control	100.0	168.7 ± 4.6 ($n = 5$)	4.8 ± 0.6 ($n = 5$)	8.2 ± 2.5 ($n = 3$)
P_i	102.3 ± 4.1 ($n = 3$)	171.8 ± 8.2 ($n = 3$)	96.8 ± 2.8 ($n = 3$)	10.2 ± 1.2 ($n = 3$)
PP_i	103.0 ± 5.2 ($n = 3$)	173.1 ± 8.1 ($n = 3$)	107.0 ± 1.9 ($n = 3$)	9.9 ± 3.5 ($n = 3$)
8-Amino-cADPR	128.8 ± 3.0 ($n = 4$)	167.4 ± 5.6 ($n = 3$)	114.4 ± 9.2 ($n = 3$)	10.5 ± 5.6 ($n = 3$)
Ins(1,3,4,5) P_4	108.7 ± 2.5 ($n = 3$)	167.6 ± 3.8 ($n = 3$)	100.0 ± 1.6 ($n = 3$)	8.5 ± 2.8 ($n = 3$)

Table 7 Differential effects of nucleotides on Ca^{2+} release induced by 1 μM Ins(1,4,5) P_3 in the presence of 50 mM caffeine in permeabilized A7r5 cells

Ins(1,4,5) P_3 -induced Ca^{2+} release in the presence of 50 mM caffeine and 1 mM of the indicated nucleotide triphosphate (NTP), diphosphate (NDP) or monophosphate (NMP) is expressed each time as a percentage of the Ca^{2+} release in the absence of caffeine. Means $\pm$ S.E.M. are shown for the indicated number of independent experiments, each performed in duplicate. In the absence of added nucleotides, 50 mM caffeine reduced the Ins(1,4,5) P_3 -induced Ca^{2+} release to $8.2 \pm 2.5\%$ ($n = 3$) of the control value.

Base	Ca^{2+} release (%)		
	Ins(1,4,5) P_3 + NTP	Ins(1,4,5) P_3 + NDP	Ins(1,4,5) P_3 + NMP
Adenine	79.3 ± 6.2 ($n = 3$)	66.3 ± 5.5 ($n = 3$)	32.4 ± 3.2 ($n = 3$)
Hypoxanthine	22.0 ± 3.5 ($n = 3$)	23.4 ± 6.3 ($n = 3$)	18.2 ± 5.2 ($n = 3$)
Guanine	26.0 ± 4.6 ($n = 3$)	28.0 ± 5.9 ($n = 3$)	23.2 ± 2.6 ($n = 3$)
Cytosine	22.8 ± 1.9 ($n = 3$)	22.5 ± 1.2 ($n = 3$)	18.5 ± 2.5 ($n = 3$)
Uracil	23.8 ± 0.9 ($n = 3$)	28.0 ± 3.7 ($n = 3$)	17.4 ± 5.2 ($n = 3$)

phosphates with micromolar affinity (Figure 4). This site is not the Ins(1,4,5) P_3 -binding domain, which prefers Ins(1,4,5) P_3 and only binds PP_i and nucleotides in the millimolar range [24], and also is not the stimulatory adenine nucleotide-binding site. An important observation was the fact that cADPR strongly modified the properties of the Ins(1,4,5) P_3 -binding site but did not bind to the site itself. Although at this moment we can only speculate on the mode of action of cADPR, an interesting hypothesis would be that cADPR, like for example Ca^{2+} , converts the Ins(1,4,5) P_3 receptor from a low-affinity high-conductance state into a high-affinity low-conductance state [31,32].

Micromolar concentrations of cADPR were needed to inhibit Ins(1,4,5) P_3 -induced Ca^{2+} release in A7r5 cells and 16HBE14o-cells. Activation of the RYR in many mammalian cells also required micromolar concentrations of cADPR. For example,

sustained $[Ca^{2+}]$ increases in pancreatic acinar cells only occurred in the presence of 100 μM cADPR [33]. The EC_{50} for Ca^{2+} release in brain microsomes was, depending on the study, 22 μM [34] and 1.5 μM [35]. Ca^{2+} stores of the nuclear envelope in liver required 10 μM cADPR to discharge [36]. Half-maximal Ca^{2+} release in Jurkat cells required 2.25 μM cADPR [37], and substantial release occurred at 7 μM cADPR [38]. Half-maximal effects of cADPR in permeabilized lacrimal acinar cells occurred at 3 μM [39]. In skeletal muscle, 17 μM cADPR released 10 times more Ca^{2+} than 1 μM cADPR [40] and 30 μM cADPR was more effective than 10 μM cADPR in PC12 cells [41]. In porcine oocytes, 200 μM cADPR induced a larger Ca^{2+} transient than 40 μM cADPR [42]. All these data indicate that the micromolar concentrations of cADPR, which are needed to see effects on the Ins(1,4,5) P_3 receptor in A7r5 cells, are in the same range as those

Figure 4 Model for the cADPR effect

The cADPR interaction site is drawn on the $\text{Ins}(1,4,5)\text{P}_3$ receptor, but may also be located on an associated protein. This domain also binds inositol phosphates (InsP_n), PP_i and various nucleotide diphosphates (NDP) or triphosphates (NTP) with micromolar affinity. This site differs from the $\text{Ins}(1,4,5)\text{P}_3$ -binding domain, which prefers $\text{Ins}(1,4,5)\text{P}_3$ (InsP_3) and only binds PP_i and nucleotides in the millimolar range. The cADPR interaction site also differs from the stimulatory adenine nucleotide-binding site of the $\text{Ins}(1,4,5)\text{P}_3$ receptor.

needed to affect the RYR in many mammalian cells. In contrast, nanomolar concentrations (EC_{50} of 18 nM) activated the RYR in sea urchin eggs [9,10]. At the moment it is not known whether the observed differences between sea urchin and mammalian systems is due to a difference in the type of intracellular Ca^{2+} channel expressed, to differences in expression of possible cADPR-binding proteins, or to modulatory factors affecting the coupling between these possible cADPR-binding proteins and the Ca^{2+} -release channel.

The inhibition of the $\text{Ins}(1,4,5)\text{P}_3$ receptor by cADPR is important for understanding the mechanism of opening and closing of this channel. Up till now, very few inhibitors of the $\text{Ins}(1,4,5)\text{P}_3$ receptor have been described. Heparin, the most used inhibitor, interacts competitively with the $\text{Ins}(1,4,5)\text{P}_3$ -binding domain [43]. Millimolar ATP concentrations similarly inhibit this site [44]. Thimerosal also inhibits the $\text{Ins}(1,4,5)\text{P}_3$ receptor at high concentrations [45], although its site of interaction still has to be determined at the molecular level. High cytosolic Ca^{2+} concentrations inhibit the $\text{Ins}(1,4,5)\text{P}_3$ receptor by bringing the receptor into a high-affinity low-conductance state [31,46]. Our present findings, that cADPR inhibits the $\text{Ins}(1,4,5)\text{P}_3$ receptor while stimulating $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding, suggest that cADPR may induce a similar high-affinity state, leading to closure of the channel. This site seems critical for the function of the $\text{Ins}(1,4,5)\text{P}_3$ receptor.

cADPR will probably not affect $\text{Ins}(1,4,5)\text{P}_3$ receptor function in the intact cell, where the [ATP] is in the millimolar range. However, cADPR is not the only metabolite acting on this site. High concentrations of nicotinic acid adenine dinucleotide phosphate (IC_{50} 200 μM) also inhibit the $\text{Ins}(1,4,5)\text{P}_3$ receptor (results not shown), raising the possibility that cADPR is perhaps a member of a broader family of endogenous inhibitors or regulators of the $\text{Ins}(1,4,5)\text{P}_3$ receptor. It is worth mentioning in this context that the proposed calcium influx factor may be a nucleotide derivative [47] and that this factor may act on a 'non-selective nucleotide site' on the $\text{Ins}(1,4,5)\text{P}_3$ receptor [48]. Although it is unlikely that cADPR will inhibit the $\text{Ins}(1,4,5)\text{P}_3$ receptor in an intact cell, the present findings are important from a biochemical point of view, since they indicate the existence of

a novel regulatory site on the $\text{Ins}(1,4,5)\text{P}_3$ receptor or on an associated protein. Therefore until other ligands interacting with this site are discovered, cADPR seems to be the only tool to further explore this site.

In conclusion, we have identified a novel regulatory site on the $\text{Ins}(1,4,5)\text{P}_3$ receptor or on an associated protein that binds cADPR and results in inhibition of the release. This site is neither the $\text{Ins}(1,4,5)\text{P}_3$ -binding domain, which prefers $\text{Ins}(1,4,5)\text{P}_3$ and only binds nucleotides and PP_i in the millimolar range, nor the stimulatory adenine nucleotide-binding site.

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